Amberless Hell Cell

Project Journal

5/28/1

- Put amberless strain on ordering doc (addgene strain 48998)
- Discovered amber suppressor tRNA mutants that recognize the UAG codon but are charged with common amino acids
- Chose to use supP (leuX mutant) because it seems to be the strongest suppressor of the amber phenotype (which means it should be transcribed and charged efficiently). (NB-in wildtype E. coli the supP mutant inhibits growth because the UUG codon coding for leucine is no longer recognized and the other tRNA has a hard time compensating, but this should not be an issue if we retain leucine CAA tRNA and add in the mutant CUA one as well).
- Will synthesize the 85bp tRNA mutant along with the 50bp upstream and downstream to make sure it is transcribed and cleaved correctly and add the prefix and suffix for BioBricking
- Will then use basicity gene recoded to use UAG for leucine and test if it works.

5/29/14

- Designed supP part to synthesize (50bp up- and downstream of the mutated tRNA along with prefix and suffix)
- Working to design a copy of the base resistance gene from Hell Cell iGEM project to use UAG to encode leucine (along with E.coli promoter and RBS)
- Would it make sense to explore the evolutionary impact of cells in the environment (i.e. not amberless E.coli cells) that would take up the modified Hell Cell genes? Would we see if cells that developed mutant tRNAs would be selected for? Directed evolution?

5/30/14

- Comment from Raman. Do we want to use the same strain but modified so that it has fewer spontaneous mutations? http://www.addgene.org/49018/
- Ben: Good idea, put this strain on ordering doc instead

6/3/14

- Ben: Finished designing the two constructs to test in the amberless hell cell (which I have put into the folder). These are variants of the serine deaminase enzyme that allows cells to grow in basic conditions, and I have included 1 or 5 UAG codons in place of leucines. Appended onto the end is the supP amber suppressing tRNA mutant, which has an CTA anticodon (which recognizes UAG) and is still charged with leucine. This is the most efficient amber suppressor known today, and I have included 100bp upstream and 50bp downstream in order to properly splice and fold the tRNA (and to include the promoter). Primers were also designed to amplify the whole sequence and the individual parts.
- Raman: Thanks for uploading the constructs Ben. I looked at them and have two comments:
    - 1) I think you would want a prefix and suffix for the serine deaminase and for the supP so that we can eventually biobrick in each separately. Would it be more efficient/cheap to make two separate DNA synthesis orders or one with both like you did here (ask Kosuke)?
        - Ben: I talked to Kosuke, and he said we can just design primers later on to add these portions in later. Otherwise when we digest the whole segment will be cut.
    - 2) Before working directly with the hell cell genes, it would be valuable to test the amber suppressor alone by GFP with UAG codons in place of leucines. That way we can first prove with an easy fluorescence assay that supP works in this system.
        - Ben: Great idea, I designed a sequence for this amber-GFP and uploaded it to the doc. Maybe this means we should only order one sdaB?

6/23/14

- Digested and ligated the supP+GFP construct into linearized pSB1C3 and transformed it into chemically competent E. coli.
- We also transformed E. coli with parts from the distribution kit (promoter+RBS and GFP+terminator) so that we can ultimately miniprep this and create a promoter-RBS-GFP-terminator construct to use as a positive control for our constructs (if the wild type E. coli don't show fluorescence we need to know that it was due to the UAG stops, not the promoter, etc.). The GFP transformed bacteria from the first day of lab

training fluoresced, meaning that it is likely that the promoter we used in our construct worked, but we still need an exact positive control.

6/24/14

- Conducted colony PCR with 30 cycles: 3 colonies of the GFP+supP construct, and 1 each of the promoter and GFP-terminator.
- Gel showed that all were negative except for possibly the promoter construct. It is too small to differentiate between insert and no insert.
- Redid colony PCR with 26 cycles and 8 colonies of the GFP+supP construct

6/25/14

- Ran the gel and found that colony 1 had a band at 700bp and colony 3 at around 1200-1300bp. The rest of the colonies were negative. These are not exactly the right sizes for the construct, but we will submit for sequencing to better understand the size discrepancy.

7/15/14

- Analyzed sequencing data for biobricked constructs:
    - GFP 7 has the proper insert with two stop codons, but one Mutation of Y->C
    - All tRNA constructs have a 12bp deletion at the 3' end of the sequence after the tRNA coding region. This was present since the biobrick PCR reaction. Might not prevent expression since it is buffer space, but we will confirm with the amberless cells.
    - tRNA 3 and 5 have the construct and each has a different substitution mutation 20-30 bp from the anticodon. Possibly to reduce toxicity.
    - SdaB 7 has correct construct!
    - SdaB + tRNA4 has the correct construct minus the deletion at the end of the tRNA
- Prepared 100 aliquots of NEB 5α competent cells
    - Conducted transformation with control plasmid to test their competency

7/16/14

- Conducted a test of the mutation screen PCR protocol I developed (see Protocols).
    - The negative control original DpsMP and MntH genes had no PCR product, which is good. The five mutation PCR product lanes have smears, so now will run the plasmids by themselves to compare.
- Competent cells from yesterday are competent. Colonies grew from control plasmid transformation.

7/17/14

- Ran plasmid product side by side with screening PCR product and there is a difference. However, the smear suggests that PCR conditions have to be optimized.

- Colony PCR of D1 and D2 mutation colonies. Picked 6 colonies from D1 and the only 2 from D2. All picked colonies were bright green due to GFP co-expression. Used D1 and D2 mutation screen reverse primers with melting temp of 56, so set annealing temp to 54C. May remove smear.

7/23/14

- Met with Ivan to discuss radiation resistance genes
    - uracil DNA glycosylases are a good bet, haven't been biobricked before and are present in every D. radiodurans species assayed
    - uvsE also a good choice, as it is not found in E. coli but in all radiation-resistant species (http://www.ncbi.nlm.nih.gov/gene/1799540)
    - can start DNA extraction from *Deinococcus radiodurans*, gram positive, soak in 95% EtOH for 5 min before adding lysozyme
    - test mntH in a solution with extra manganese added
    - make sure to use competent cells that aren't recA-
    - would it be worthwhile to look at promoters for DNA damage (SOS response, etc. http://parts.igem.org/wiki/index.php?title=Part:BBa_J22126 http://en.wikipedia.org/wiki/SOS_box) to do radiation biosensors?
- Made TGY plates and liquid media for *D. radiodurans*

8/1/14

- Prepared cryostocks of SdaB_amber Clone7, tRNA Clone5, and SodCu/Zn.

8/4/14

- Attempted amplification of uvsE, DR0689 (needs a better name), and DR1751 from *D. radiodurans* genomic DNA. The primers have the biobrick ends on them. Used Earl thesis for some PCR parameters:
    - 60 ng genomic DNA/reaction (Earl suggested 20 ng but I said YOLO)
    - 1 uM primer (5 uL of 10 uM stock/reaction)
    - 25 ul AmplitaQ gold in a total reaction volume of 50 uL
    - 1 min of 95C denaturation and 45 sec of annealing; 34 cycles
    - Ran 3 tubes with just each primer mix, no DNA. Ran 1 tube with just DNA
- Ran the PCR products on a gel to see if it was successful
    - Gel shows that the right genes for all 3 were likely PCRed out
- GFP_amber Clone7, Sdab+tRNA_amber Clone4, tRNA Clone3 grown out for miniprepping and cryostock preparation tomorrow

Let's give these genes some names!
DR0689 = Dudg1 (D. radiodurans uracil-DNA glycosylase 1)
DR1751 = Dudg2 (D. radiodurans uracil-DNA glycosylase 2)

Amberless Hell Cell TODO list while Raman is gone. Parts 1 and 2 can be done in parallel, so you guys might want to figure out who works on what.

Part 1: Getting genes out of *D. radiodurans*

- If uvsE, Dudg1, and Dudg2 PCR amplification works from genomic DNA, proceed with restriction digest with E + P and ligate into the pSBC13 for biobricking.
    - Colony PCR and sequence verify the insert before proceeding.
    - We have SODCu/Zn already biobricked in pSB Amp plasmid. The cryostock is in the -80C. Please radiation test this along with the other 3 genes.
    - Test the properties of each of these 4 genes when transformed into wild type E. coli. Talk to Ivan to set up a radiation resistance assay to characterize the genes.
- MntH and DpsMP primers arrived late, so I couldn't include them in the genomic DNA PCR with the rest. Please try the same protocol from 8/4/14 for gene amplification from genomic DNA. Modify if it didn't work.
    - The primers have the biobrick ends, so if the PCR is successful, digest, ligate and transform wild type E. coli with them.
    - Colony PCR using the primers DscreenF + VR (for Dps) and MscreenF + VR (for MntH)!! Then sequence verify.
    - Test the radiation resistance of these two genes in wild type e. coli
- If none of the genes get PCR'ed out, you might need to test whether we actually have D. radiodurans DNA. Get the D. radiodurans 16S rRNA primers from Ivan and run our genomic DNA with that, then sequence.
    - If we don't have the right DNA, then we have to regrow the bacteria and try genomic DNA extraction again.
- I doubt we'll get to this point, but next thing will be to mutation PCR in a stop codon into each gene. Already designed the mutation primers for Dps and MntH. Design them for the other 3 genes using this and my geneious files for Dps, MntH to help:
  http://openwetware.org/wiki/Richard_Lab:Site_Directed_Mutagenesis
    - Perform mutation PCR and sequence verify that you put in a stop codon for leucine codon.

Part 2: Test our amberless proof of concept in the amberless *E. coli*

Use http://www.sciencemag.org/content/342/6156/357.abstract and its supplementary methods section if you have any questions regarding the handling of amberless. I think they grow best in the lower 30C incubator.

- Grow a liquid culture of the amberless in LB + Zeocin (25ug/ml), cryostock it!!
- Make LB + Zeocin and LB + Chlor + Zeocin plates for keeping a plate stock of the amberless (let's not lose it!) and for transformations respectively. Use same 25ug/ml concentration of zeocin.

- Figure out how to make them competent by talking to Kosuke/Kendrick/iGEM guide to galaxy. I think we may need to manually make them competent or do electroporation. Test transforming amberless by using a really easy GFP plasmid from the parts distribution.
- Now simultaneously, miniprep the GFP_Amber biobrick and tRNA clones 3 and 5 biobricks. Digest and ligate into pSB1C3 plasmid.
- After confirming that you can transform the amberless, transform wild type (NEB 5alpha) and amberless cells with the ligation product.
    - Compare how many colonies transform.
    - Are any of the colonies green?
    - Sequence 2-3 green amberless colonies
    - Grow the green amberless colonies and one or two of the wild type colonies to prove that we express GFP only in amberless and not in regular E. coli.

8/5/14

- Started overnight culture of D. rad because the genomic DNA looked cloudy (although PCR did work successfully)
    - (Raman) - Don't think we need more DNA. The cloudy white thing is likely a precipitated protein contaminant that isn't effecting anything. Just spin it down and use the liquid portion. Anyways, the genes we wanted got PCRed out so that confirms we got the right genomic DNA.
    - (Ben) - Yeah, I set it up before the sequencing results came back. Everything looks fine now.
- PCRed out MntH and DSPMP from genomic DNA, cleaned up, and ran on a gel (looked good)
- Sent these two samples, along with uvsE, Dudg1, and Dudg2, off for sequencing
- Still waiting on zeocin

8/7/14

- Zeocin in. Plates made. Colonies started. Use 50% salt in LB for Zeocin. also, use .5 μL/mL Zeocin
- MntH, dudg1, dudg2,dpsmp, uvse digested with E and P, as was linear psb1C3. Will ligate and transform tomorrow
- Amberless cells made electrocompetant using normal protocol on iGEM guide to the galaxy
- Made lb+zeocin plates and lb+zeocin+chlor plates to grow amberless cells

8/8/14

- MntH, dudg1, dudg2,dpsmp, uvse ligated and transformed into regular 5-alpha e. coli on chlor. Plates left on bench at RT for weekend.
- Transformed competent amberless cells with 5M in the 2014 distribution kit just ot see if the cells could be transformed.

    - Used electroporation at 1800 V
- Made cryostock of amberless cells from liquid cultures from 8/7

8/11/14

- MntH, Dudg1/2, DPSMP, and UvsE transformations produced colonies, performed colony PCR to verify inserts, colony pcr results strange, will show you (Raman) tomorrow. Made overnight culture of one of each to possibly sequence tomorrow
- Amberless cells transformed with very high efficiency
- Ran colony PCR and everything appeared to have correct insert.
- Plated 5 alpha cells on lb+zeocin+chlor plate and untransformed amberless on lb+zeocin+chlor to confirm that only transformed amberless will grow on these plates.
    - This was confimed. Nothing grew on these two plates

8/12/14

- 5 radiation genes were grown up overnight and miniprepped, sent off for sequencing (colony PCR results were strange but I want to know what is wrong, so I sent off one colony from each for sequencing, if these come back good we can start mutagenesis).
- PCRed the GFP with tRNA IDT construct using gBlock primers
- Digested, ligated with chlor backbone and transformed with electroporation into amberless cells
    - used 1800 V
    - grew at 30 degrees overnight

8/13/14

- Sequencing results looked shitty, but this was probably a result of the primers (VF2 and VR haven't been working for anyone), can resend for sequencing with primers used to amplify them from D. rad DNA if we want
- Nothing grew on the transformed amberless plates
- Redid ligation and transformation
    - Electroporated at 1950 V and plated all cells and incubated overnight at 30 degrees

8/14/14

- Three colonies express GFP!
- Made liquid cultures of each colony and left plate at 30 degrees to grow more

8/15/14

- Liquid cultures didn't grow well
- The plate has more expressing colonies
- Will remake liquid cultures next week and give them more time to incubate

8/18/14

-Made liquid cultures of 4 fluorescing colonies in morning

8/19/14

-Miniprepped 4 colonies and submitted colonies 3 and 4 for sequencing

-Transformed colony 4 into amberless and into 5 alpha cells for control

-Electroporation at 2400 V for amberless

-Heat shock for 5 alpha cells

8/20/14

-One GFP colony grew in 5 alpha cells

-GFP lawn grew in amberless

-Sequencing for 4 came back as a bad read

-Sequencing from 3 looks good except for a 12 base pair deletion of the tRNA

8/21/14

-Transformed amberless and 5 alpha with plasmid 4 since sequencing was good

-amberless electroporation at 2200 V

-5 alpha heat shock

8/22/14

-Amberless plates grew very good GFP expressing colonies

-Nothing grew on the 5 alpha plate

-Redoing control with electrocompetent cells

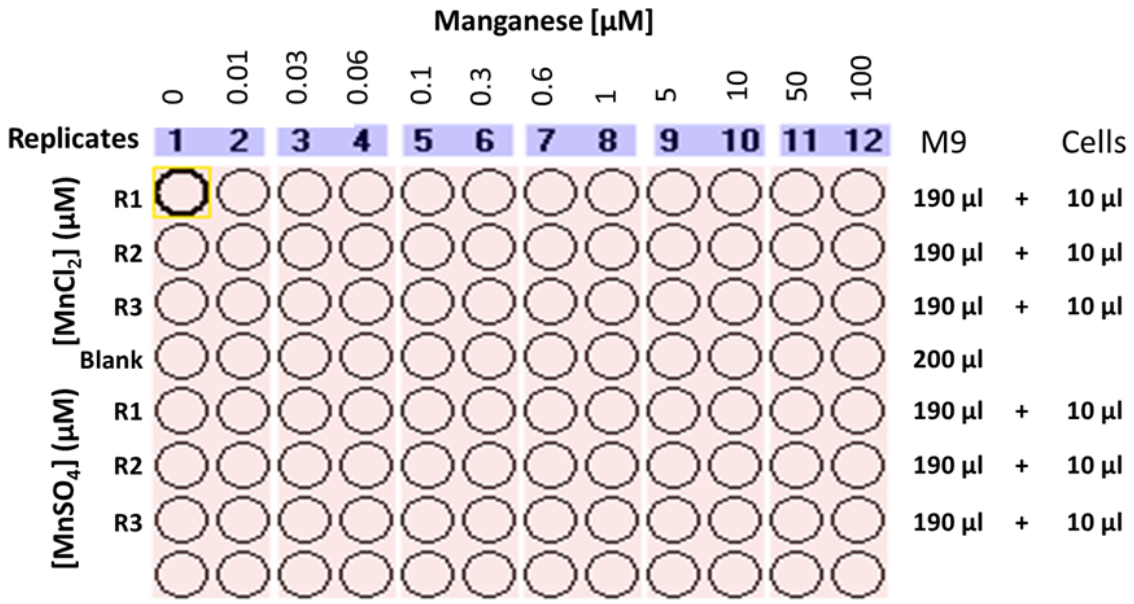
-electroporating at 2200 V to replicate amberless transformation protocol

9/24/14

Set up plates for MntH and uvsE manganese growth assay

There was no significant difference in OD600 absorbance of blanks with different manganese concentrations, so I will use 3 replicates of the same blank - M9 without manganese.

Growth curves at different Mn concentrations and 30C incubation



9/25/14
-PCR amplifying IDT Amber-aeBlue construct
1 uL (10ng) template DNA
25 uL Q5 2X Master Mix
1.25 uL wasp-ampli F+R mix (10uM)
22.75 uL Milli-Q water
30 cycles at Ta=66C Product length ~ 1200bp, 40s of extension

PCR cleanup using Qiagen MinElute Reaction Cleanup Kit

9/26/14
-Digest aeBlue+tRNA construct with EcoRI + PstI for biobricking and testing expression
2 uL CutSmart buffer
0.2 uL EcoRI
0.2 uL PstI
4 uL DNA (250 ng/ul)
13.6 uL H20
-After digestion, did PCR cleanup and got 14ng/uL final concentration

10/1/14

-Started growth experiment with varying concentrations of Mn at 30C (see 9/24). OD600 measurements every 1 hr.

Ligation with Insta sticky end

10/2/14
- Ran a colony PCR: Picked 8 clones of amberless cells with aeBlue+tRNA, 4 clones of amberless with tRNA, 2 clones each of the equivalent in 5alpha
- Gel extracted pSB1C3 digested with EcoRI + PstI, MntH mut1 and mut2 with EcoRI + PstI, Dudg1 and Dudg2 with XbaI + PstI

10/3/14
- Ran colony PCR gel and got positive amberless cells with the construct, but no clear positives for 5alpha
    *Liquid culturing aeBlue+tRNA clones 1, 7, 8; tRNA clone 1, 2, 4; 5alpha tRNA 1 for
    sequencing

-DpnI digested mutation PCR of UvsE mut1 and mut2
-Insta sticky end ligated MntH mut1 and mut2 with pSB1C3 for biobricking
-Transformed 5alpha cells with the MntH mut1,2 biobricks (chlorR) and the UvsE mut1,2 (ampR)

10/10/14

-Transformed 5alpha and amberless chemically competent cells with 10 ng plasmid DNA from aeBlue+tRNA clone 7, which was sequence verified to have the correct construct. These were transformed under the exact same conditions, both plated on LB+Chlor and one extra LB+Chlor+Zeocin to see if the extra antibiotic impairs the amberless expression of aeBlue.
-Picked 3 different clones from each UvsE Mut1 and Mut2 plates. Growing 3mL overnight cultures in LB+Amp
-Picked one very blue colony from the aeBlue+tRNA biobrick plate, now called clone 9. Growing one liquid culture in LB+Chlor and another in LB+Chlor+Zeocin to compare blue protein expression. This is being grown at 37C because the amberless seem to grow better at that temperature compared to 30C.

1. Transform amberless and NEB 5alpha with aeBlue+tRNA 7 construct
    1.1. Pick 1 blue clone from each and liquid culture for protein extraction
    1.2. Stain with his tag or flag tag
2. Sequence Uvse mut1 and mut2
    2.1. Compare radiation resistances versus wild type

2.2. If time, digest and ligate in the tRNA
2.3. Compare radiation resistances again

10/15/14

-Amberless transformed aeBlue plate has many deep blue colonies but 5alpha has no blue colonies. It looks like the amber stops with tRNA construct only works in amberless cells!

-Inoculated:

-70 mL cultures of a blue amberless clone and 5alpha clone from the plate to grow at 30C for 2 days for Western blotting

-30 mL cultures of a blue amberless clone2 and 5alpha clone2 from the plate to grow at 37C for 2 days for Western blotting